A MORPHOLOGICAL AND PHYSIOLOGICAL INVESTIGATION OF THE SELF-INCOMPATIBILITY OF THE ORLANDO TANGELO

By

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INTRODUCTION

Citrus breeders have found that certain varieties in the mandarin group, Citrus reticulata, often produce high quality progeny when used in breeding programs. Furthermore, one of these varieties, 'Clementine' tangerine, is valuable to breeders because it is monoembryonic and also produces some monoembryonic progeny.

Interspecific hybrids with mandarin parentage have been introduced and some, such as the 'Orlando' and 'Minneola' tangelos, have been found to be sexually self-incompatible and to yield erratically in solid plantings in the field (62, 87). The 'Clementine' tangerine has also been shown to be self-incompatible (93). A cross between the 'Orlando' and the 'Clementine' produced the 'Robinson' which is both monoembryonic and self-incompatible (105, 106).

Because breeders are continuing to use these monoembryonic and self-incompatible varieties and their progeny in breeding programs (18) it is likely that more self-incompatible varieties will be introduced in the future. It is unlikely that high quality varieties will be discarded solely because they are self-incompatible. Therefore, fundamental information about self-incompatibility in citrus is needed so that the problem may be corrected by some means other than cross-pollination.

Knowledge of the morphological and physiological nature of sexual incompatibility in citrus is very sparse; however, there has been work conducted with other plants that indicates several types of incompatibility may occur. In addition, evidence exists that in some plants there is a

relationship between pollen germination, pollen tube elongation, sexual fertilization and naturally occurring growth substances.

It was the purpose of this study to determine the nature of sexual incompatibility in citrus through a morphological study of pollen germination and pollen tube growth and a physiological study of concurrent changes in natural growth substances in the pistils of 'Orlando' tangelos.

LITERATURE REVIEW

Flowering Characteristics

In the United States the main flowering period for citrus is in the early spring (8). Because of differences between varieties and local environmental conditions the flowering period in a given area often appears to extend over a considerable interval of time.

Flower bud initiation for the main flowering period has been reported by a number of workers (1, 6, 34, 53, 100) to take place with the spring flush of growth.

Investigators (48, 104, 107, 115) have shown that less than 7 percent of the flowers of some citrus varieties developed into mature fruit. These varieties were 'Lisbon' lemon, 'Valencia', 'Hamlin', 'Pineapple' and 'Shamouti' sweet orange.

The types of inflorescence in the sweet oranges have been discussed by Reece (104) and Zacharia (142). In general, there may be cymose or racemose inflorescences or combinations of these types on the same flowering branch. Reece described the inflorescence as "leafless", if the growth consisted of one or more flowers without leaves, and "leafy", if the flowers were axillary on new, leafy shoots. Forms in which the development was chiefly floral but with only one or more leaves present on the stalk on which the flowers were located were also classed as "leafy" inflorescences. It was found that the leafy inflorescence consistently produced a higher percent of fruit set than the leafless inflorescence.

Both Zacharia and Reece noted that the apical bud of the flowering shoot set the greater number of fruits with a decreasing number set at each succeeding node distal to the apical bud.

Reproductive Processes

The citrus flower has been described (8) as composed of more or less united sepals (calyx), a whorl of showy petals (corolla) which are white in most species, a whorl of stamens and a pistil consisting of a stigma, style, and superior ovary resting upon a disk or nectary. The ovary ultimately develops into the mature fruit.

The stigma is larger in diameter than the style and its epidermal cells are comparatively long, papillose, unicellular hairs which secrete a viscous fluid that aids in retention and germination of the pollen (8). According to Bartholomew and Reed (8), Klimenko, in Russia, reported that the stigma of the sweet orange was receptive to pollen for a period of 6 to 8 days after anthesis, with the greatest receptivity shown at the time of the initial opening of the flower. The stigmas of the sweet lime (22) and several varieties of lemon (102) were also found to be most receptive the day of anthesis even though they had attained maturity the previous day.

The cylindrical style is traversed by stylar canals that pass downward from their opening on the surface of the stigma to the locules in the ovary (8). There is one stylar canal for each locule. Upon germination of the pollen the tubes grow through the canals to the ovules. The cells lining the canals are secretory and similar to those on the surface of the stigma. Alternating with the stylar canals are vascular bundles. The style abscisses from the ovary at an early stage after the flower has opened.

The ovary develops into a mature fruit composed usually of 10 to 12 carpels filled with juice vesicles and seeds (8). The young ovary is characterized by central placentation with each locule bearing anatropous ovules, the micropyles of which face the axis of the ovary (32). The ovule has outer and inner integuments surrounding the nucellus that form a micropylar opening. It is attached to the central axis by the funiculus.

Osawa (94) has described the development of the embryo sac in Poncirus trifoliata, 'Washington' navel orange and 'Satsuma' mandarin. The mature embryo sac was found to consist of 8 nuclei. These nuclei were the egg and 2 synergids, the 2 polar nuclei, and 3 antipodals. At anthesis the embryo sac was usually in the 2- or 4-nucleate stage and reached the 8-nucleate stage several days later.

Osawa (94) found the stages of pollen development were in advance of those of the embryo sac. By the time the primordia of the inner and outer integuments of the ovule had appeared, the pollen mother cells were already in the second nuclear division or even in the tetrad stage. When the megaspore mother cell was in synapsis, the pollen grains had already formed. Before the anthers dehisced, the nucleus of the pollen grain divided forming the generative and vegetative nuclei. Frost (32) pointed out that it was not known whether the division of the generative nucleus and formation of 2 sperm nuclei took place before or after pollination.

In Citrus and its near relatives the haploid number of chromosomes is 9 and species and generic crosses are not uncommon (63). Most forms are assumed to be quite heterozygous because there is considerable variability among gametic progeny (32).

Webber (128) pointed out that because of the attractiveness of citrus flowers to insects, ample self-pollination was seldom a problem. He suggested that self-pollination in many varieties could take place without the aid of insects because of the close proximity of the anthers to the stigma.

Frost (32) has described pollen germination and pollen tube growth in citrus. When the pollen contacts the stigma it is retained by the thick, sticky secretion on the surface of the papillose hairs. Here it absorbs moisture and the pollen tubes soon develop. The pollen tubes enter the stylar canals, which are open to the surface of the stigma, and grow downward by way of these canals to the locules in the ovary, passing through a sticky secretion like that of the stigma. The tubes grow through the placental hairs and enter the micropyle of the ovules, penetrating the nucellus at the apex of the embryo sac.

Osawa (94) reported that in P. trifoliata fertilization occurred almost 4 weeks after pollination; however, he was judging the time from full bloom and since the trees could have been flowering over a period of several weeks, a precise time interval could not be determined. According to Frost (32), Strasburger and Toxopeus found that fertilization occurred about 3 or 4 weeks after pollination in several species of citrus. Coit (15) cited Ikeda as having found that the pollen tube reached the ovule 30 hours after pollination and that fertilization occurred in from 48 to 72 hours after pollination. He did not indicate the species with which Ikeda had experimented.

Frost (32) cited the work of Toxopeus in which he reported a 27-chromosome endosperm in 2 diploid forms of citrus after fertilization had occurred, which indicated that double fertilization had taken place.

Osawa (94) noted that subsequent to fertilization the embryo sac and the ovule underwent considerable elongation. This was concurrent with development of the endosperm. The endosperm nuclei appeared to divide simultaneously throughout the length of the sac and were scattered in the cytoplasm. The cytoplasm formed a thin layer along the inner periphery of the embryo sac and, even though the endosperm nuclei continued to divide, this free-nucleate stage persisted for a comparatively long time. Osawa's drawings showed that cytokinesis had taken place 60 days after full bloom. Cell walls formed in the endosperm tissue at the micropylar end of the sac; however, a large vacuole remained from the center to the chalazal end of the sac. The endosperm was considered to be a temporary nutritive tissue which was eventually absorbed by the developing embryos.

Osawa (94) found that the zygote of P. trifoliata did not divide until 3 or 4 weeks after fecundation and then both the first and second divisions were transverse. Strasburger, cited by Frost (32), reported that development of the zygote was uninterrupted in southern European countries, while Schacht, also cited by Frost, found that in Madeira a particular mandarin variety showed the first division about 2 months after flowering.

According to Frost (32), apomictic or nucellar embryos are commonly formed among many citrus varieties. Of nucellar embryony Frost writes:

> Since nucellar embryos develop asexually by ordinary mitotic division of cells of the nucellus, no male cells contribute to their formation, and no reduction division occurs in the seed-parent cells which give rise to them. Nucellar seedlings, therefore, not only inherit from the seed parent alone, but are actually identical with it in genetic constitution, except for possible differences due to somatic (bud) variation.

Osawa (94) reported that the mode of development of nucellar embryos in P. trifoliata was similar to that found by Strasburger in C. aurantium. While the zygote was still dormant, a few large nucellar cells near the

top of the embryo sac were easily distinguished from the adjacent nucellar cells by their larger nuclei and denser cytoplasm. Some were deeply seated in the nucellar tissue. At nearly the same time as the first division of the zygote these nucellar cells divided repeatedly and formed a number of embryos. These nucellar embryos were distinguished from the gametic embryos by their irregular shape and great variety in size, as well as by the absence of a suspensor. Biermann, cited by Frost (32), concluded that at an early stage the nucellar embryos were usually less advanced than the gametic embryo; however, Toxopeus (also cited by Frost) found that most of the nucellar embryos were multicellular at the time the zygote began to divide.

Bain (7) studied the morphological, anatomical, and physiological changes in the developing 'Valencia' orange fruit. She found 3 distinct stages in fruit development, which were designated as Stage I, Stage II, and Stage III. Stage I, the cell division period, lasted from 4 to 9 weeks, depending on the time of flowering. Stage II, the cell enlargement stage, was the period of maximum fruit growth. Stage III was the maturation period and was distinguished from Stage II by decreased rates of morphological, anatomical, and physiological changes, and by the general process of ripening. Ford (30) reported that meristematic activity continued in various parts of the ovary of 'Eureka' lemon after the petals and sepals had abscissed.

Unfruitfulness in Some Citrus Varieties and Hybrids

Most standard varieties of citrus are fruitful either because the normal reproductive processes, which are required for fruitfulness, take place or because they are parthenocarpic. However, there are exceptions (129).

The 'Washington' navel and various other navel oranges fruit erratically. The navels are seedless because their ovules abort before sexual fertilization can take place. They are erratically fruitful because they are weakly parthenocarpic.

Some of the tangelos, interspecific hybrids of grapefruit and tangerines (129), also fruit erratically.

Robinson (108) reported that the 'Orlando', 'Minneola', 'Thornton', and 'Yalaha' tangelos were "more or less" sterile to their own pollen because their fruits were quite seedy when grown in mixed variety plantings but in solid block plantings the fruit were relatively seedless. He recommended that these varieties be planted in solid blocks to lessen the likelihood of seediness. He was unaware of the self-unfruitfulness which was later reported in the 'Minneola' (87) and 'Orlando' (62). Krezdorn and Robinson (62) reported that both hand-pollination experiments and field observations showed the 'Orlando' to be seedless and unfruitful because of sexual self-incompatibility; however, they reported that seedless crops frequently occurred in solid plantings due to weak or erratic parthenocarpy.

Similarly, Oppenheimer (93) in Israel and Soost (117) in California reported that the 'Clementine' tangerine fruited poorly due to self-incompatibility but frequently produced seedless crops because of a weak parthenocarpy.

Reece and Gardner (105) reported that 'Robinson', a hybrid of 'Clementine' and 'Orlando', was seedless, or few-seeded, when self-pollinated and set relatively few fruits. These were hand-pollinated tests and the degree of parthenocarpy under field conditions was not ascertained.

Burnawa (13), in Russia, pollinated sweet oranges, mandarins, and lemons with pollen from the same plant, pollen from different plants of

the same variety, pollen from different varieties of the same species, and with pollen from plants of other species in the same genus. From these pollinations he concluded that cross-pollination with pollen from different varieties of the same species, and with pollen from plants of other species in the same genus gave the highest fruit production, had the fewest abortive ovaries, and also produced larger fruit.

Sexual Self-incompatibility

Self-fertility in higher plants is the capacity to produce viable seed from the union of gametes when self-pollinated and therefore implies self-fruitfulness, although self-fruitfulness does not always require self-fertility. Conversely, self-sterility is the inability to produce viable seed following self-pollination. Self-sterility may be the result of self-incompatibility, which is the inability to combine gametes.

The location in the pistil in which the incompatibility reaction takes place varies with different plants (112). Germination of the pollen grain on the surface of the stigma and the subsequent process of pollen tube penetration may be inhibited. Germination of the pollen may be normal but the growth of the pollen tube through the conducting tissue of the style inhibited. Pollen tubes may reach the ovules and penetrate the embryo sac but union of the gametes does not take place or the fertilized egg cell or young embryo aborts.

The studies of some investigators (11, 95) have shown that there is a relationship between the number of nuclei in the pollen grain and the site of inhibition. In those genera having inhibition in the stigma, the pollen grains were trinucleate. Genera in which inhibition was related to the growth of the pollen tube had predominantly binucleate

pollen grains. Brewbaker considered it probable that the incompatibility inhibition seldom occurred until after the second mitotic division.

Various genetic theories for the different types of incompatibility reactions have been reviewed by Stout (120). The simplest, and perhaps most common type, is the oppositonal factor hypothesis of East and Mangelsdorf (26), in which incompatibility is determined by genes, designated by the letter "S", that form a multiple allelomorphic series as S_1, S_2, S_3, ---S_n. Any 2 of these may be carried by the plant. Pollen cannot function in the style of a plant carrying the same alleles as the pollen. This results in either failure of the pollen tubes to reach the ovary or their reaching it after the ovules have degenerated.

Incompatibility reactions that take place on the surface of the stigma are not as common as those which take place after germination of the pollen grain and extension of the tube. According to Gardner et al. (36), Muller found that in Oncidium flexuosum and a number of other species of orchids the pollen was injurious or poisonous to the stigma, causing it to turn brown and to decay prematurely. At the same time, unpollinated stigmas remained fresh. When pollen from other plants was placed on the stigmas there were no signs of injury and fertilization and fruit set ensued. The pollen that acted injuriously upon stigmas of its own flowers functioned satisfactorily on other plants. Yasuda (136, 137, 138) found that the placenta of Petunia violacea secreted a "special substance" which diffused into the style and retarded or completely inhibited germination of its own pollen and the development of pollen tubes, but permitted pollen of other varieties to function.

Sears (112) found that incompatible pollen of Brassica oleracea and Raphanus sativus seldom germinated after self-pollination. When it

did germinate the tubes never penetrated the stigma. Tatebe (122, 123, 124) reported the same to be true for Japanese radish and that even compatible pollen was slightly inhibited. Both Sears and Tatebe were able to overcome the inhibition in the stigma by removing a thin layer of stigmatic tissue before pollination. They concluded that the inhibition was limited to the layer of tissue removed. However, in the self-incompatible guayule, Gerstel and Riner (38) reported incompatible pollen tubes were unable to penetrate beyond the epidermis of the stigma and, unlike the cases reported by Sears and Tatebe, removal of the inhibiting tissues of the stigma did not facilitate growth of the pollen tube because germination of the pollen took place only on the papillae of the receptive stigma.

Addicott (2) reported that pollen germination and growth of the pollen tube are not necessarily related phenomena because they may be stimulated independently or simultaneously. Sears (112) noted that in most self-sterile plants pollen germination was not affected, but growth of the pollen tubes was retarded.

Knight (58) studied the problem of self-incompatibility in the apple and found that even though the pollen tubes attained sufficient length in artificial culture to reach from the stigma to the egg, in vivo germination was slow on the stigma and growth of the tube in the stylar tissue was not sufficient to reach the egg in time for fertilization to take place.

Cooper (16, 17) and Weeks and Latimer (130) also found self-incompatibility in apple was due to slow pollen tube growth. Each noted that initial growth seemed equal. Later, growth of the selfed tubes slowed down while the tubes resulting from cross pollination grew on to

the ovules. Cooper reported that either the growth of the tubes in the self-pollinated pistils was retarded or the growth of the tubes in the cross-pollinated pistils was accelerated by some factor or factors. Some investigators (23, 25) have reported that in Nicotiana, the tubes grew at a steady or even slightly accelerated rate following selfing but did not reach the ovary in time to fertilize the egg. When cross-pollinated, the tubes grew at a continuously accelerating rate.

According to Eyster (29), pollen of the 'Golden Rose' petunia germinated and tubes grew into the neck of the style but seldom extended as much as half the distance from the stigma to the ovary before the style abscissed. Slow pollen tube growth has also been reported by Dorsey (24) as the cause of self-incompatibility in plums.

Oppenheimer (93) confirmed the assumption that the self-incompatibility of 'Clementine' tangerine was due to slow pollen tube growth. He found that when self-pollinated the tubes had not penetrated into the style after 9 days, having been delayed in the tissue of the stigma. Pollen tubes from 'Duncan' grapefruit were found in the lower portion of 'Clementine' styles after 3 days. In 6 days fragments of 'Duncan' tubes could be found near the ovules. However, Oppenheimer pointed out that his observations were somewhat inadequate due to unsatisfactory techniques.

In 1926 Frost (31) observed that citrus varieties with good pollen set seeds about as readily from selfing as from crossing. In 1928, however, Nagai and Tanikawa (cited by Frost, 32) checked 28 varieties of citrus and found 4 of them to be self-incompatible, although they had good pollen and were very or moderately seedy when cross-pollinated. Torres, in 1932 (cited by Frost, 32) reported that certain pummelos native to Thailand were seedless when not cross-pollinated, but their pollen

was effective in producing seeds when used to pollinate other varieties. Wong (134) found a number of varieties of citrus were less seedy when self-pollinated than when open-pollinated. He explained that this might be due to lethal and sub-lethal effects from incompatible recombinations and therefore represented a degree of self-incompatibility.

Krezdorn (59) has reported that the sister 'Orlando' and 'Minneola' tangelos were both self-incompatible and cross-incompatible with each other. Furthermore, the 'Seminole', another sister variety, was self-compatible and cross-compatible with both 'Orlando' and 'Minneola'. Reece and Register (106) found that 'Robinson', a hybrid of 'Clementine' and 'Orlando', was self-incompatible.

Sears (112) has found that among many plants having self-incompatibility characteristics there is a great variation in the amount and manner of tube growth. In some plants the incompatible tubes may penetrate only short distances into the styles, in others the tubes penetrate farther into the style, and in still other plants the incompatible tubes may reach the upper portion of the ovary or the vicinity of the ovules before growth stops. Modlibowska (82) found in apple and pear that even though the incompatibility reaction completely arrested the tubes in the style, the time and place varied and depended in part upon temperature.

In Nemesia strumosa (112) incompatible tubes grew the length of the style at the same rate as compatible tubes. At the bottom of the style the growth of the incompatible tubes suddenly decelerated and came to a stop at the top of the ovary. Sears also found that in selfed Tolmiea menziesii the pollen tubes were inhibited in the lower area of the style. Only a small percentage of the tubes that reached the bottom of the style proceeded into the ovary. Of the few tubes that entered the ovary fewer

yet reached the ovules. He concluded that if ovules are fertilized they are limited to the upper half of the ovary.

In the Chinese chestnut McKay (81) found that when self-pollinated the pollen tubes grew down through the style and into the cavity of the embryo sac. Male gametes were seen adjacent to the egg and fused polar nuclei but syngamy was not observed. He concluded that the male gametes and the eggs were incompatible. Sears (112) reported the incompatible tubes of Gasteria grew normally and fertilized the ovules but that the ovules degenerated, while cross-fertilized ovules continued to grow.

Pearson (97, 98) was able to overcome self-incompatibility in cabbage by bud pollination made from 1 to 5 days before anthesis. The increased fertility from bud pollination was attributed to the longer interval available for the pollen tubes to reach the ovules before the latter degenerated. Attia (4) concluded that the increase in fertility of bud-pollinated cabbage was due to the absence, or insufficient concentration, of an inhibiting substance in the young styles rather than to the increased length of time afforded the pollen tube to traverse the style. He proposed that the inhibiting substance increased up to anthesis. Attia made no attempt to extract or identify the substance; however, he suggested that it was probably secreted in the style when the flower bud reached a certain physiological maturity.

According to Tatebe (122) self-pollination 2 days before anthesis or 2 days after anthesis in the Japanese radish resulted in seed production but pollen germination was inhibited if applied the day the flower opened. He postulated that the inhibiting substance in the surface stigmatic tissues which caused self-incompatibility was not present when the flower

was in the bud stage and that there was a decline in activity of the inhibiting substance in the older flowers. Similar successful results with bud-pollination have been reported by Sears (112).

In a study on bud-pollination of Japanese persimmon, pear, plum, and peach, Asami (3) found that in all cases fruit set and seed production was reduced. Pollinations were made only 1 and 2 days prior to normal opening of the flowers.

Sanclemente-Pineda (110), experimenting with self-incompatible cacao, concluded that incompatibility was due to an inhibiting substance which delayed development of the pollen tube. He was able to destroy the substance with applications of either hydrogen peroxide or potassium permanganate to the pistil. When these compounds were applied to the stigmas the pollen germinated and normal tube extension took place. Similarly, applications of Vitamin B_1 plus a hormone were successful. Naundorf (88), also working with cacao, found that extracts from incompatible ovules inhibited germination and growth of pollen tubes in agar. He also found that the same extract from incompatible ovules stimulated germination and tube growth of pollen from self-fertile trees.

Eyster (29) reported that when flower buds of 'Golden Rose' petunia were self-pollinated, just as they were beginning to develop anthocyanin in the petals, fertilization occurred. An ovarian extract of the 'Golden Rose', transferred to the stigma and style of cross-compatible varieties, rendered those varieties incompatible to the 'Golden Rose' pollen.

Linskens (68) was of the opinion that the incompatibility reaction strongly localized on the surface of the stigma of some plants in the Cruciferae was due to specific compounds formed under the influence of incompatibility alleles. He suggested that these compounds diffused from

their point of origin in the interior of the stigma to the surface and blocked germination and tube growth. However, Linskens (70) and Heinen and Linskens (50) later reported that the pollen did not germinate because of insufficient moisture on the stigma. Sears (112) noted that where pollen germination was affected by incompatibility, the stigmatic cells did not secrete appreciable amounts of fluid.

Linskens (70) and Heinen and Linskens (50) postulated that pollen from cross pollination liquified and made the cuticle of the stigma permeable by activating or releasing cutin-splitting enzymes (cutinases). Thus, there could be a biochemical mechanism by which the tips of the pollen tubes were prevented from penetrating the cuticle of the surface of the stigma. If the cuticle was removed prior to pollination pollen tube growth proceeded unrestricted.

In Forsythia, Geissman (37) cites Kuhn as having found that difficulty in the crossing of 2 varieties was associated with the presence of rutin or quercitrin in the pollen, each of which prevented pollen germination. Each variety had in its stigma an enzyme that would hydrolyze only quercitrin or rutin but not both. Thus, the pollen could germinate only on styles containing the appropriate enzyme.

There is general agreement among investigators that pollen tubes secrete enzymes while passing through the tissues of the style and the presence of several such enzymes and coenzymes has been established (56).

In Linskens' (68) studies of the biochemical reaction that took place when growing tubes were inhibited in the style he found glucoproteidic compounds formed in the conducting tissue which were not formed with compatible combinations. There was also a disturbance of the normal carbohydrate metabolism. He assumed that the system of inhibiting substances that were produced either blocked the growth of the tube in a way

similar to an antigen-antibody reaction, or disturbed the carbohydrate metabolism, or both, by way of enzyme mechanisms. Linskens (68, 70) outlined 3 possible working mechanisms for incompatibilities in the style:

> 1. After an incompatible pollination substances are formed or activated in the conductive tissue of the style that act as inhibitors of the growth of the pollen tubes. In that case the proteid-carbohydrate complexes found should be indicated nomenclaturally as iso-antibodies.
>
> 2. Under the influence of the bipartite S-locus, the growing pollen tube forms antigens that induce specific antibody formation in the conductive tissue of the style. The antibodies react with the existing surplus of antigens. The reaction product blocks enzyme systems that lead to abnormal polymerization of carbohydrates on one hand and to disturbance of nuclear metabolism on the other.
>
> 3. Already during the development and the formation of the male gametes in the anthers an active exchange of substances has taken place with the female organs (carpels, style). At that time the style antibodies are already formed against pollen-tube antigens. Because there is still no surplus of antibody material, precipitate formation does not yet occur. When, however, after self-pollination tube formation takes place at last and connected with this the formation of superfluous antigens, the antibodies already present in the cells of the conductive tissue react with the antigens; a precipitate is formed with all further consequences of the inhibition.

Linskens (68, 69) made serological tests of extracts of pollen and styles of Petunia hybrida by injection into rabbits to produce antiserums. Precipitation tests of sexual combinations showed that all homologous combinations reacted strongly while heterologous combinations were much weaker or entirely absent. It was considered from this that the inhibition of the pollen tube growth was a disturbance of the metabolic relation between sexual partners before fertilization.

There are numerous instances in the literature in which investigators have attempted to study and correlate the chemotropic responses of pollen tubes in vitro with compatibility reactions that occur in vivo. However Johri and Vasil (56) concluded that the wide range of responses with

a large number of species of plants makes it unwise to draw any conclusions from this type of experimental evidence alone. In a study of pollen-tube chemotropism in Lilium, Rosen (109) found that positive reactions with a variety of pistil-pollen combinations in vitro were independent of the compatibility of these combinations in vivo.

While investigating the nature of fruit thinning with naphthaleneacetic acid (NAA) on apple trees, Luckwill (73) discovered that it was possible to induce incompatibility between the pollen tubes and stylar tissue of normally cross-compatible types by aqueous sprays of NAA.

Eyster (29) was able to overcome the self-incompatibility in the 'Golden Rose' petunia by spraying the flowers, shortly before self-pollination, with alpha-naphthalene acetamide. By application of naphthalene acetamide plus pollen, Emsweller and Stuart (27) were able to overcome self-incompatibility in Lilium.

Mashkin (80) made successful self-pollinations on cherry, pear, apple, and quince by dusting the stigmas with indoleacetic acid (IAA) immediately after pollination.

Addicott (2) reported the effect of 33 pure growth substances on the germination and growth of pollen of Tropaeolum (a dicotyledon) and Milla (a monocotyledon). Several classes of these substances showed activity, including vitamins, plant hormones, and pyrimidines and purines. Singh and Randhawa (116) induced greater pollen tube length in vitro in 2 mandarin varieties by application of gibberellic acid to the medium in which the pollen was incubated. However, this is not evidence that gibberellic acid could be used to overcome incompatibility.

According to Johri and Vasil (56), Tanaka suggested that the slow growth of the pollen tube of Pinus densiflora was due to the presence of inhibitors in its own pollen.

The X-irradiation of pollen enabled Lewis and Crowe (66, 67) to overcome self-incompatibilities in breeding work with apple, cherry, and pear. They explained that by inducing the incompatibility gene to mutate, a pollen grain containing such a mutant-allele was then compatible even in self-pollination because it had an allele which was different from those in the style. Therefore, on selfing there could be a few seeds produced which were the result of "S" gene mutation. Since thousands of pollen grains could be placed on a stigma, self-incompatibility provided a sieve for selecting out of millions of pollen grains only those which had an "S" mutation.

In recent years there have been numerous reports on the activity of plant growth-promoters and -inhibitors. This has developed chiefly because of the availability of new techniques for the separation of chemical components of plants through the use of paper chromatography and the development of biological assay methods. Lewis (65) reasoned that the delaying effect of compatible pollination on abscission in cherries was probably an auxin effect. He suggested an explanation for the effect of alpha-naphthalene acetamide on self-incompatibility in African marigolds, cabbage, and red clover as reported by Eyster (29). Lewis (65) postulated that incompatibility involved many different and highly specific reactions between the pollen and the styles and that a single substance which would counteract these reactions would be affecting something fundamental to the whole process. Luckwill's (73) induction of self-incompatibility in apples with sprays of naphthaleneacetic acid is an indication that growth substances may play a role in incompatibility reactions.

Relation of Pollination to Fruit Set and Parthenocarpy

Sexual self-incompatibility prevents the formation of seeds from the union of gametes after self-pollination; however, the presence of the pollen tubes growing in the pistil may influence the development of parthenocarpic fruit (46).

Parthenocarpy has been defined by Gustafson (45) as the development of seedless fruit without sexual fertilization. However, there are fruits such as mangosteen that produce embryos in ways other than by fertilization of the egg. Furthermore, seedlessness and parthenocarpy are not synonymous because seedlessness may result from embryo abortion subsequent to fertilization.

As Gustafson (46) pointed out, the presence of pollen on the stigma is seldom a sufficient stimulus for promoting the development of an ovary but if the pollen germinates and produces a tube, reaching some distance into the pistil, the chances of a fruit being formed are much greater. This has been clearly shown by Yasuda (138, 139, 140) and Yasuda et al. (141). These workers found that egg-plant flowers pollinated with Petunia violacea grew into seedless fruits. Ovaries of Capsicum sp. were stimulated to grow into fruits when the flowers were pollinated with a species of Physalis. Pollination of Nicotiana sp. with pollen from Petunia violacea resulted in seedless fruits, but pollen of egg-plant had no such stimulating effect. It was seen, however, that pollen tubes of Petunia grew faster than those of egg-plant in the style of Nicotiana. The latter pollen tubes made only slight growth. When cucumbers were pollinated with their own immature pollen or pollen several days old or with pollen from other cucurbitaceous plants, fruits sometimes developed

and these were always without seeds. Fruits were formed only from pistils in which pollen tube growth occurred. They determined that the pollen tubes had to reach the base of the style or the top of the ovary before the ovary would be stimulated sufficiently to develop into a fruit.

Working with Melandrium, van Overbeek et al. (126) reported that enlargement of the ovary and ovules was induced by pollination and was usually well under way before the pollen tube reached the embryo sac. Yasuda (139, 140) also noted that when Petunia pollen grew in the pistils of egg-plants and Nicotiana sp. ovary development had begun by the time the pollen tubes reached the ovaries. Other workers (54, 99) reported a slight increase in fruit set in the parthenocarpic 'Washington' navel orange following cross-pollination, thus implying a stimulation from the growth of pollen tubes. No studies were made of the pollen tube growth.

Gardner et al. (36) have cited many more examples in which pollination or pollen tube growth has influenced the development of ovarian and other tissue connected with the fruit. One such example was that found in orchids in which the ovules could not complete their development and reach the stage for fertilization unless pollination occurred first.

Yasuda (139, 140) found water extracts of Petunia pollen when injected into the ovaries of egg-plants and tobacco flowers stimulated growth. Similar results were obtained when a water extract of cucumber pollen was injected into cucumber ovaries (141). In no instance were seeds produced. Gustafson (40) was able to obtain enlargement of the ovary, and in some cases the production of full-sized fruits when chloroform extracts of pollen were applied to the pistils of a number of plants.

Laibach (64) found that there was a substance in hot water extracts of the pollinum of orchid flowers and pollen of Hibiscus which, when applied to the stigmas of orchid flowers, could cause swelling of the ovaries. These same extracts also caused elongation of oat coleoptiles.

An extractable growth-promoting substance obtained from pollen of corn by Wittwer (131) was not active when applied to tomato ovaries, but was capable of inducing elongation of bean seedlings. He postulated that the function of the pollen growth hormone was concerned with stimulating pollen tube growth because corn styles are very long, thus requiring extensive pollen tube growth. In this same study an extractable growth substance was found in young corn kernels that was specific for fruit set and fruit development but was not capable of inducing elongation in bean seedlings.

Growth hormones have been found in pollen tubes of Nicotiana and Antirrhinum by Muir (83). Fukui et al. (33) examined the acid and neutral fractions of ether extracts of corn pollen. Three growth promoting zones occurred on chromatograms of the acid fraction, 1 of which was evidently IAA. Two areas of promotion were found on chromatograms of the neutral fraction. Luckwill and Powell (77) concluded that a growth-promoting substance found in the acid fraction of ether extracts of apple pollen was not indoleacetic acid because it did not give characteristic color reactions even though it did separate at nearly the same R_f as indoleacetic acid on paper chromatograms. He referred to this substance as Malus auxin 2.

Gustafson (40) suggested that initiation of growth in the ovary is caused by an increase in the growth-hormone content of the ovary as a

result of pollination. According to his concept, pollen tubes transfer the hormones from the pollen into the ovary together with the sperm nuclei. Other workers have suggested that the amount of auxin in the pollen would not be sufficient to cause ovary growth. They postulated that pollen might supply an enzyme which would release auxin from a precursor. The auxin thus supplied to the ovary, either directly or indirectly as a result of pollination, would act as a trigger to set off growth reactions.

After examining the auxin content of pollen, pollen tubes, and pistils before and after fertilization, Muir (83, 84) found pollen tubes contained much more auxin than pollen alone. He also observed an increase in the auxin content of the pistil following pollination. Muir detected an enzyme system in the pollen that could release auxin from its bound form in the tissue of the pistil. When the concentration of the hormone was sufficient, growth of the ovary was initiated and development of the fruit commenced. Within a few days after pollination some of the hormone migrated toward the basal end of the pistil and from there possibly moved into the pedicel of the flower, preventing formation of the abscission layer. However, Muir drew attention to the fact that this initial growth in the ovary usually ceased unless fertilization also had taken place.

In 2 varieties of Nicotiana tabacum, Lund (78) demonstrated that during the first few hours after pollination the hormone concentration increased in the apical half of the style and then decreased later as the concentration increased in the basal half of the style. He found that the peak of the hormone concentration in any part of the style was closely correlated to the location of the apices of advancing pollen tubes. At the time pollen tubes were seen entering the ovaries there was also a correlated increase in the hormone content. By chromatography he showed

that this hormone was probably indoleacetic acid. To account for this migrating gradient of auxin, Lund (79) found an enzyme secreted by the growing pollen tubes that converted tryptophan into active indoleacetic acid and thereby initiated fruit development.

Fertilization and Auxin Production

Following fertilization in the tomato, Murneek (85) reported that there was a marked increase in metabolism and stimulation of plant growth. Avery et al. (5) found that at the time of pollination the total auxin present in corn kernels was extremely small. Immediately after pollination the auxin content increased sharply, reaching a peak in from 1 to 3 weeks. Wittwer's (131) findings with corn kernels agreed with the results of Avery and his co-workers. Further investigation of the auxin content in corn kernels by Haagen-Smit et al. (47) showed the free auxin extracted with 95 percent ethyl alcohol consisted chiefly of indoleacetic acid. They were of the opinion that this auxin possibly had a role in the embryo development and thus fruit set. However, since indoleacetic acid applied to the tissue was relatively ineffective in fruit set, they thought its presence could not account for all the stimulatory effects that usually accompanied pollination and subsequent fertilization.

Nitsch (89, 90) noted that fertilized achenes of strawberry contained relatively large amounts of free auxin whereas the receptacle yielded no free auxin. He also noted that only fertilized achenes imparted active growth stimulation to adjacent areas of the receptacle. The auxin concentration in the achenes was found to increase sharply about 12 days after pollination.

Auxin and Fruit Set

Luckwill (74) was of the opinion that even though workers had reported successful attempts to induce fruit set by application of synthetic growth-promoting compounds the majority of the attempts were unsuccessful and not reported in the literature. Some of the earliest reported successful attempts to induce parthenocarpic fruit set were by Gustafson (39, 41), Gardner and Marth (35) and Wong (133, 134) on a variety of plants and with a variety of chemicals.

Lewis (65) postulated that only many-seeded fruits are responsive to attempts to induce parthenocarpy by synthetic growth substances. This theory was contradicted by the reports of Rebeiz and Crane (103) and Crane et al. (20) who were able to induce parthenocarpy in 'Bing' cherry and 'J. H. Hale' peach.

Luckwill (74) was of the opinion that auxin was the primary factor involved in the initial stimulus to fruit set and growth following pollination and fertilization. Furthermore, he considered that this auxin had its origin in the developing seed.

Many workers have supported this idea by finding extractable growth promoters in the seeds of a variety of plants. In some early work Tukey (125) found that mechanical destruction of the embryo of peach and cherry fruits before the transition from Stage II to Stage III in the growth period resulted in a cessation in fruit development and subsequent abscission. Stahly and Thompson (119) found that high auxin levels in 'Halehaven' peach ovules coincided with rapid endosperm and embryo development. Avery et al. (5), Haagen-Smit et al. (47) and Hinsvark et al. (51) found indoleacetic acid prominent in developing corn kernels while Stowe et al. (121) reported

indoleacetic acid and 2 other Salkowski-reacting substances were synthesized by the tissue of corn endosperm grown in vitro. Gustafson (42) found a high auxin content in ovules of squash and tomato. Murneek (86) stressed the importance of the endosperm and associated tissue in seed and fruit development. Luckwill (71, 72) reported that an auxin build-up in apple seeds coincided with the development of the endosperm. A variation in auxin production could be correlated with 3 periods of fruit drop. Nitsch (89, 90) found a large increase in auxin content in strawberry achenes 12 days after pollination.

From their observations of gibberellic acid-induced growth of parthenocarpic fruit in 'Bing' cherry and 'J. H. Hale' peach, Rebeiz and Crane (103) and Crane et al. (20) postulated that the growth patterns of parthenocarpic fruit and seeded fruit were so similar that growth of these fruits probably was not controlled by auxins originating in the seeds. Both Crowe (21), working with apples, and Crane et al. (19), working with figs, concluded that in these fruits the factors controlling fruit enlargement were most likely not auxinic. Crowe suggested that after syngamy a factor "X" was produced by the fertilized ovule that controlled fruit development.

Gustafson (42) made a study of the auxin content in flowers of varieties of sweet orange, lemon and grape. The auxin content was considerably higher in the ovaries of those varieties of oranges and lemons that were seedless and supposedly did not require pollination than those varieties in which pollination and fertilization were required. He found the same to be true for seedy and seedless grapes. In a comparison of fruitful and barren selections of 'Montmorency' cherry, Gustafson (44) found that the hormone content was much higher in the pistils of the

fruitful selection than the barren selection. From this he postulated that sufficient concentration of auxin was present to initiate the growth process in those plants where pollination for fruit development was unnecessary. In the cherry there was a positive correlation between fruit set and the growth hormone content of the ovary of the flower.

MATERIALS AND METHODS

In 1962 this experiment was conducted with trees in an 8-year-old 'Orlando' orchard. The trees were on rough lemon rootstock. Because of freeze injury to these trees the following winter, the 1963 experimental material was obtained from 15-year-old 'Orlando' trees on Cleopatra mandarin rootstock. These trees had lost only a few leaves as a result of the freeze.

Treatments

1962.-On 6 trees near the center of the orchard, 2200 flowers were depetaled and emasculated just prior to anthesis. Half of the flowers on each tree were self-pollinated and half were cross-pollinated with 'Duncan' pollen. The pollinated flowers were labeled with numbered tags and other flowers on the same shoot removed. Only leafy inflorescences were used. The pistils were not bagged or covered since there was little likelihood that a bee would visit a depetaled and emasculated flower and because wind pollination in citrus is negligible. Furthermore, the changes in the microclimate of the pistil resulting from bagging possibly could have introduced appreciable error.

1963.-The basic procedure used in 1962 was followed but several modifications were made.

First, 90 non-pollinated flowers were depetaled, emasculated, and tagged similarly to those which were selfed and crossed.

Second, because of the information obtained in 1962, fewer flowers

were needed. Only 550 each of self-pollinated and cross-pollinated flowers were used.

Morphological Studies

Both selfed and crossed 'Orlando' pistils were collected 0, 1, 2, 4, 6, 8, 10, 12, 14, and 19 days after pollination in 1962. In 1963 the collections were made 0, 4, 8, 12, 16, and 40 days after pollination. Part of the pistils were fixed in FAA and others in Craf's solution (55).

In order to study the growth of the pollen tubes, stained squashes of the pistils collected at each date were examined under the microscope.

In preparing the squashes considerable difficulty was encountered in softening and clearing the ovaries and styles.

The use of chloral hydrate (28) and pectinase (96, 114) permitted only limited study of pollen tube growth. Chloral hydrate resulted in excessive hardening of the tissues and pigmentation interfered when pectinase was used.

A satisfactory procedure was obtained by slightly modifying a method described by Yap (135). This method could be used only with the material fixed in FAA. Material fixed in Craf's solution could not be softened.

The epidermis of the ovaries was removed with a razor blade leaving the ovules and central axis exposed. The pistils were then split longitudinally and cleared with repeated washings of 10 percent NaOH at 70 to 75° C. This required from 0.5 to 1.5 hours.

If the pistils were not divided in half lengthwise before clearing they would invariably shrink during the process. Temperatures above 75° C. caused shrinkage while temperatures below 70° C. resulted in poor clearing.

After clearing, the pistils were rinsed in water at 70 to 75° C.,

hydrolyzed for 10 minutes in normal HCl at 70 to 75° C., rinsed in water at room temperature and allowed to soak in 4 percent NH_4OH for 15 minutes. Material left overnight in this solution was usable but did not stain as well as that used immediately.

After staining in lacmoid, the pistils were quartered with dissecting needles on a microscope slide. A drop of 1 percent NH_4OH was placed on the pistil sections. The sections were then squashed under a cover slip. The slide was prevented from drying with drops of 1 percent NH_4OH or the lacmoid stain. Usually, 10 minutes' staining time in lacmoid was sufficient; however, longer periods were sometimes required.

The lacmoid stain was prepared by dissolving 10 mg lacmoid (Resorcin Blue) in 5 cc water. At the time the stain was used it was brought to pH 8 with drops of 1 percent NH_4OH.

Callose plugs in pollen tubes that were growing in the style stained a very bright blue (Figure 5). The tube walls proper took no stain and remained transparent. However, in the ovary the pollen tubes usually (but not always) stained a faint light blue. This permitted easy differentiation from the placental hairs, which might otherwise have been confused for pollen tubes. Callose in the sieve tubes of the vascular bundles also stained blue and caution had to be taken not to confuse the sieve tubes with pollen tubes.

Progress of the pollen tube in the ovule and penetration of the embryo sac was also observed in paraffin sections. Ovules were dissected from the ovaries that had been fixed in Craf's solution. Dehydration was carried out in tertiary butyl alcohol (55). The material was then imbedded in Tissuemat and subsequently sectioned with the rotary microtome at from 8 to 15 microns in thickness. The sections were stained in

Delafield's hematoxylin (111). The slides were mounted in either Diaphane or Permount.

Physiological Studies

Selfed and crossed 'Orlando' pistils were collected 0, 2, 4, and 8 days after pollination in 1962. In 1963 collections of selfed, crossed, and non-pollinated pistils were made 0, 4, 8, 12, and 16 days after pollination. Samples were collected between 10 A.M. and noon on each date. The pistils were frozen immediately after collection by dropping into test tubes immersed in a dry ice-acetone bath. They were then held at 0° F. until extraction.

The ether-soluble free auxins and inhibitors in the acid and neutral fractions of methanol extracts of styles and ovaries were separated by means of paper chromatography. Biological activity was analyzed by the wheat straight-growth test according to the procedure of Hendershott (49). Results of these tests were then compared with the morphological observations of the pollen tubes to determine if a correlation existed.

Samples weighing from 0.5 to 2.0 gm fresh weight were ground in cold methanol (91) with a "Virtis 23" grinder for 2 minutes. After grinding, the samples were extracted in 50 ml of methanol for 2 hours at 0° F.

The sample was then filtered and the filtrate reduced to dryness in a vacuum distillation apparatus with distilling flask at 40° C. The dry sample was taken up with distilled water and adjusted to pH 2.8 with concentrated HCl. This aqueous phase, the acid and neutral fractions of the extract, was filtered into a separatory funnel. The ether soluble fraction was separated by vigorously shaking in 3 separate 50 ml aliquots of peroxide-free diethyl ether. The aqueous phase was discarded and the

ether phase was dried with anhydrous sodium sulfite. The sodium sulfite was removed by filtering and the ether-soluble fraction was reduced to dryness and re-dissolved in a definite volume of methanol. Sub-sample aliquots representing 0.25 gm fresh weight of material were taken down to dryness and re-dissolved in ether.

The sample was streaked evenly in a narrow band across a 1.5-inch strip of Whatman No. 1 filter paper 18 inches long. Samples of more than 0.25 gms fresh weight were found to deposit excessive quantities of oils and waxes on the streaked band and uneven partitioning and streaking of the chromatogram always resulted.

Equilibration and development of the chromatograms was carried out in a glass tank, 12 x 12 x 24 inches, at a constant temperature of 78° F. The chromatograms were equilibrated for 16 hours in a water vapor atmosphere. The developing solvent, isopropanol-ammonia-water (10:1:1 v/v/v), was then added. Development was by descending chromatography. The solvent front advanced to approximately 20 cm past the original streak in 7.5 hours, at which time the chromatograms were removed from the tank and dried.

The dry chromatograms were observed under ultraviolet light (3660 Å) and the areas of fluorescence marked and recorded. The area of the original streak was then delineated and the remaining developed area divided into 16 equal sections parallel to the original streak. One section, of the same dimensions as each of the 16, was marked off in the area of solvent run above the original streak. This was designated the control section for the subsequent bioassay test.

Some chromatograms were treated with Ehrlich's reagent (5 gm p-dimethylaminobenzaldehyde/50 ml of concentrated HCl, diluted 1 to 4 with acetone before using) for the detection of indole compounds (113).

The presence of growth-promoting and growth-inhibiting substances on the chromatograms was determined by bioassay using the wheat coleoptile straight-growth test (92). Glass-distilled water was used throughout this test.

'Atlas 66' wheat seed was soaked in water for 2 hours and planted shallowly in trays of vermiculite. The trays were kept in the dark for 72 hours at 78° F. At the end of this time seedlings between 25 and 35 mm in length were selected for the test.

The individual wheat coleoptiles were sectioned under a dim green light using Thimann's coleoptile microtome. A 4 mm section, 3 mm below the tip and with the primary leaf included, was used in this test. The coleoptile sections were floated in distilled water for 2 hours prior to incubation in the test solution.

Each of the 18 previously marked sections of the chromatograms, including the control, were inserted into a shell vial containing 1 ml phosphate-citrate buffer (pH 5.0) with a 2 percent sucrose concentration (92). Five wheat coleoptiles were placed in each vial and the vials placed on a wheel revolving at 1 rpm. Incubation was in the dark for 20 hours at a constant 78° F. At the end of this period the coleoptiles were removed and measured by means of an eyepiece micrometer in a binocular microscope.

Known concentrations of synthetic IAA were dissolved in methanol, streaked on paper, and developed and assayed in the same manner as the actual samples. The values obtained were then used to plot a standard curve for IAA equivalents.

Known quantities of synthetic IAA were ground up with styles and ovaries, chromatographed, and bioassayed in the same manner as the other

samples. From this it was possible to determine what effect the constituents of the sample and the mechanics of the procedure had on the auxin.

RESULTS

Morphological

Pollen tube growth, fertilization, and subsequent ovule development in the pistils of 'Orlando' tangelo are summarized for 1962 and 1963 in Table 1.

Pollen of both 'Orlando' and 'Duncan' germinated equally well on 'Orlando' stigmas and the resulting pollen tubes of each grew to the base of the style and upper ovary at equal rates. Growth of the 'Orlando' pollen tubes was inhibited at the base of the style or upper region of the central axis of the ovary and the tubes were never seen extending the remaining distance to the locules and the ovules. This was true both in 1962 and 1963.

Following cross-pollination in 1962, 'Duncan' pollen tubes were seen in the ovules on the eighth day (Figure 1) and in the embryo sac (Figure 2) on the twelfth day. Multinucleate endosperm was observed on the fourteenth day after pollination (Figure 3). There was little further change in the contents of the embryo sac through the nineteenth day.

In 1963 the 'Duncan' pollen tubes had penetrated the ovules within 4 days and multinucleate endosperm was observed on the eighth day after pollination. On the fortieth day after pollination, approximately 5 weeks after the first evidence of fertilization, the zygote had not undergone its first division (Figure 4). At this time cytokinesis had not commenced within the endosperm, nor was any active nuclear division observed in the

area of the nucellus where formation of nucellar embryos would be expected to take place.

Callose deposits were abundant in the pollen tubes in the upper one-third of the style (Figure 5) following both self- and cross-pollination. Their numbers were greatly diminished in the mid-portion of the style, and were seldom seen in the basal one-third of the style. In the ovary the pollen tube diameter was slightly increased and the tube walls usually stained a faint light blue. There were no distinguishing characteristics in the pollen tubes between self- or cross-pollination.

The fresh weight of the styles of unopened flowers was the same in both 1962 and 1963 (Table 2). The increase in fresh weight during the first 4 days after pollination was similar in both years.

Abscission of the styles began 8 days after pollination in 1962 (Table 1). In 1963 abscission of the styles was completed by the eighth day.

The ovaries from unopened flowers were about equal in size in both 1962 and 1963 (Table 2). However, the growth of ovaries following pollination in 1962 was more rapid than in 1963. On the eighth day after pollination in 1962 the ovaries were almost twice as large as they were on the corresponding date in 1963 (Table 2). In 1963 the weight of the cross-pollinated ovaries was greater on all sampling dates than either self-pollinated or non-pollinated ovaries. The weight of the self-pollinated ovaries was greater than the non-pollinated ovaries on each date.

In the first year there was no noticeable ovary abscission during the 19 days of the sampling period. However, in 1963, there was a period of ovary abscission that began between 4 and 8 days after pollination and extended to the period between 12 and 16 days after pollination (Figure 6).

Because the ovary numbers were also being reduced through sampling, the percentage of abscission between sampling dates was calculated on the number remaining after the previous sampling.

The percentage abscission was approximately 5 times greater for self- and non-pollinated ovaries (52 percent) than for cross-pollinated (12 percent) between the fourth and eighth day. There were no non-pollinated ovaries remaining after the eighth-day collection; however, there was a smaller number of the non-pollinated flowers at the beginning of the experiment.

Ninety-six percent of the self-pollinated and 57 percent of the cross-pollinated ovaries remaining on the trees 8 days after pollination had abscised before the twelfth-day collection. There was not a sufficient number of self-pollinated ovaries for sampling the twelfth day. Abscission of cross-pollinated ovaries dropped to 23 percent between 12 and 16 days. No abscission occurred between 16 and 40 days.

It was noted that during the period of ovary abscission all the tagged ovaries on the trees had a light green color and appeared very weak. By the sixteenth day after pollination, when ovary abscission had stopped, the color of the ovaries had turned a darker green and appeared to be stronger.

There was a difference in minimum and maximum temperatures during the pollination and sampling periods between the 2 years (Table 3). The recorded mean Fahrenheit temperatures in the area for the day of pollination and the 9 days following averaged approximately 12° lower in 1962 than in 1963.

The trees used as a source of material in 1963 had endured subfreezing temperatures 3 months earlier, which caused about 5 percent of the leaves to fall.

TABLE 1.--Pollen tube growth and ovule development in the pistil of the 'Orlando' tangelo after pollination in 1962 and 1963.[a]

Days after Pollination	1962		1963	
	Self-pollination	Cross-pollination	Self-pollination	Cross-pollination
1	P.t. growing into style	P.t. growing into style	-	-
2	P.t. in middle 1/3 of style	P.t. in middle 1/3 of style	-	-
4	P.t. in base of style and upper ovary	P.t. in base of style and upper ovary	P.t. in base of style and upper ovary	P.t. in embryo sac
8	No further p.t. growth Styles beginning to abscise	P.t. in ovules Styles beginning to abscise	No further p.t. growth Styles abscised	Developing end. Styles abscised
12	Styles abscised	P.t. in embryo sac Styles abscised	-	Developing end.
14	-	Developing end.	-	-
16	-	-	-	Developing end.
19	-	Free multinucleate end. Zygote undivided No nuc. emb. formation	-	-
40	-	-	-	Free multinucleate end. Zygote undivided No nuc. emb. formation

[a]Abbreviations: p.t.-pollen tubes end.-endosperm nuc. emb.-nucellar embryo.

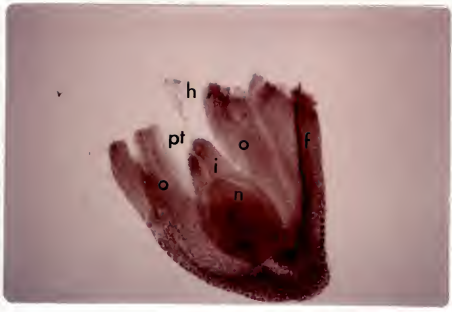

Figure 1. Pollen tube in 'Orlando' ovule after cross-pollination. This stage observed 8 days after pollination in 1962 and 4 days after pollination in 1963. (100X). pt, pollen tube; h, placental hairs; i, inner integument; o, outer integument; n, nucellus; f, funiculus.

Figure 2. Embryo sac in 'Orlando' ovule penetrated by pollen tube after cross-pollination. This stage observed 12 days after pollination in 1962 and 4 days after pollination in 1963. (440X). e, embryo sac; n, nucellus; pt, pollen tube.

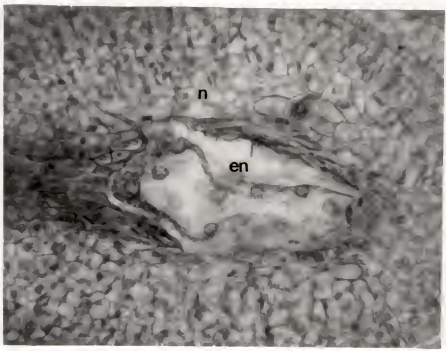

Figure 3. Multinucleate endosperm in 'Orlando' ovule after cross-pollination. This stage observed 14 days after pollination in 1962 and 8 days after pollination in 1963. (440X). en, endosperm; n, nucellus.

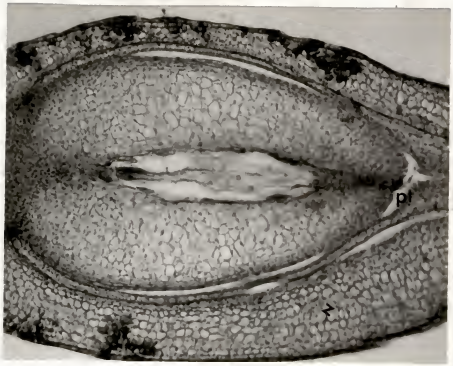

Figure 4. Undivided zygote in 'Orlando' ovule 40 days after cross-pollination in 1963. (145X). z, zygote; pt, pollen tube.

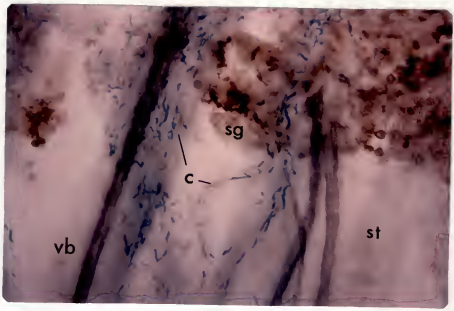

Figure 5. Blue stained callose plugs in pollen tubes in 'Orlando' style. (100X). c, callose plugs; sg, stigma; st, style; vb, vascular bundle.

TABLE 2.--Number of ovaries and styles per gm fresh weight at various dates following non-, self-, and cross-pollination.

Days after Pollination	No. Styles per gm			No. Ovaries per gm		
	Non	Self	Cross	Non	Self	Cross
1962						
0	44	-	-	50	-	-
2	-	44	42	-	44	42
4	-	35	35	-	32	32
8	-	-	-	-	19	19
1963						
0	43	-	-	51	-	-
4	38	37	38	44	37	35
8	-	-	-	38	36	32
12	-	-	-	-	-	21
16	-	-	-	-	-	15

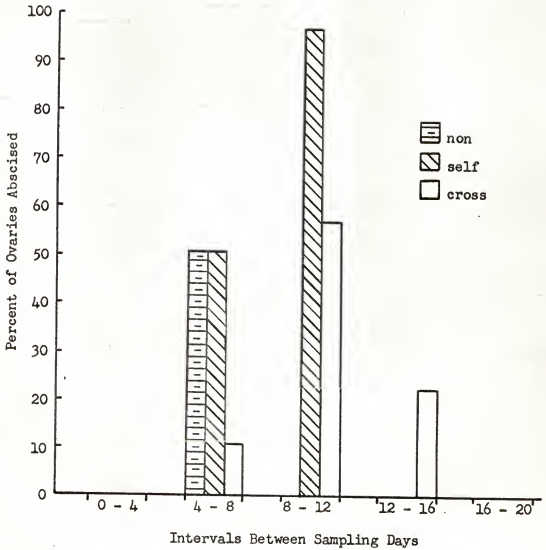

Figure 6. The percent of non-, self-, and cross-pollinated ovaries that abscised between sampling days in 1963. There were no non-pollinated ovaries after 8 days and no self-pollinated ovaries after 12 days because all had been collected or had abscised.

TABLE 3.--Maximum and minimum Fahrenheit temperatures during sampling periods in March, 1962 and 1963.[a]

March, 1962				March, 1963		
Date	Maximum	Minimum		Date	Maximum	Minimum
13	80	57		7	68	47
14	60	49		8	72	44
15	74	58		9	73	50
16	72	54	Date of	10	77	58
17	67	42	----------	11	86	61
18	73	40	Pollination	12	90	65
19	78	42		13	90	67
20	80	49		14	90	64
21	86	61		15	83	65
22	82	66		16	88	63
23	68	56		17	90	68
24	80	58		18	92	66
25	70	60		19	93	61
26	76	58		20	85	67
27	74	50		21	75	50
28	83	44		22	69	45
29	83	53		23	75	40
30	86	60		24	78	49
31	88	65		25	80	50

[a]Temperatures recorded at a climatological field station in an orchard near Clermont, Florida. Data furnished by U.S.D.A. Citrus Field Station, Orlando, Florida.

Physiological

Growth response with IAA.

Chromatographed molar concentrations of synthetic IAA were located in 4 sections ranging from Rf 0.53 to 0.83 (sections 10 to 14) with the highest concentration around Rf 0.68 (Figure 7, A). Under ultraviolet light the chromatographed IAA showed no color with 10^{-7} and 10^{-6}M concentrations, an inconsistent faint light purple with 10^{-5}M, and a light purple or light blue with 10^{-4}M. The area of fluorescence was seen at an average Rf of 0.68 but ranged slightly higher or slightly lower on different chromatograms.

A pink chromogenic reaction was observed with Ehrlich's reagent and corresponded with the areas defined under ultraviolet light. However, no reaction was obtained on chromatograms containing IAA at a 10^{-5}M or lower concentration.

When pure IAA was added to a sample of styles and ovaries and subjected to the entire fractionation procedure, the physical and chemical characteristics of this IAA on paper chromatograms was identical to that of pure IAA applied directly to chromatograms (Figure 7, B). The growth-promoting properties of the IAA were not reduced from that of similar concentrations of IAA chromatographed alone, indicating that IAA, if present in the tissue, was not being inactivated during the grinding and extraction period.

Growth substances extracted from 'Orlando' tangelo pistils.

Histograms of the bioassays of chromatographed extracts from 'Orlando' tangelo styles and ovaries are presented in Figures 8, 9, and 10. Each histogram represents the means of 2 bioassays, except for the styles in 1963, which are the means of 3 bioassays. Two areas of minor growth

promotion, one area of major growth promotion, and one area of major growth inhibition were located on the chromatograms. The 3 areas of growth-promoting activity on the chromatograms are referred to as promoter I, promoter II, and promoter III, according to their ascending R_f value. No special designation was assigned to the area of inhibition.

Promoter I.-Promoter I, R_f 0.0 to 0.12 (sections 1 and 2), occurred on the chromatograms of most samples. When present, it was usually concentrated at R_f 0.03, the section that included the original streak. Under ultraviolet light there was no conspicuous color fluorescence in this area and Ehrlich's reagent never produced a chromogenic reaction.

Promoter I appeared to be similar to an unidentified compound or compounds running on or near the starting line of chromatograms developed in isopropanol-ammonia-water which has been reported by other workers (9, 12, 21, 52, 75, 119). It has been considered an artifact by some and none have attached significance to this area of promotion. In this work no meaningful differences were found in the concentration of promoter I in extracts of styles and ovaries or between self-, cross-, and non-pollinated styles and ovaries. Promoter I was also detected when only synthetic IAA was chromatographed and the activity was comparable to that of chromatographed sample extracts. Other than the fact that this area of promotion commonly occurred, these results were considered of no significance to this work.

Promoter II.-Promoter II, R_f 0.53 to 0.83 (sections 10-14), was the major area of growth promotion. The highest concentration was centered around R_f 0.68 and was present in as many as 4 or as few as 2 sections. A slight variation in the R_f of promoter II from sample to sample was observed; however, slight variations in R_f are commonly encountered in chromatographic analyses (127).

There was inconsistent color fluorescence associated with promoter II under ultraviolet light. Either no color, light purple, light blue, or sometimes a bright blue were found in the area of promotion. The only definite reaction to Ehrlich's reagent was a pink color formed on chromatograms of cross-pollinated ovaries sampled at 16 days. This would imply that if promoter II was IAA, it had reached a concentration greater than 10^{-5}M on this date.

The concentration of promoter II on each chromatogram was determined as the summation of units of promotion above the controls in 4 sections showing the greatest promotion between R_f 0.53 and 0.83. The mean concentration for replicated bioassays of styles and ovaries is presented in Table 4 in units of promotion per 0.25 gm fresh weight.

Because the weights of the pistils in the present study increased at different rates among self-, cross-, and non-pollinated flowers (Table 2), it is more meaningful to discuss promoter II concentrations on a per-ovary or per-style basis. Fresh weight concentrations were converted to per-ovary and per-style concentrations by dividing the number per sample into the total units of growth above the controls (Table 4 and Figure 11). It has been pointed out in similar studies with tobacco (78) that in a growing organ, such as the ovary, hormone concentrations appear to remain the same when expressed on a dry-weight or fresh-weight basis, but when expressed on the basis of the individual ovary, there may be seen a net synthesis of hormone.

There were no large differences between self- and cross-pollination in the concentrations of promoter II in the styles (Table 4). There was, however, a higher endogenous level of promoter II in the self- and cross-pollinated styles 4 days after pollination in 1962 than in similar samples

in 1963. In 1963 the concentration of promoter II in the non-pollinated styles 4 days after pollination had dropped to one-third of the original level at pollination and was lower than the concentration in self- or cross-pollinated styles.

In both self- and cross-pollinated ovaries in 1962 the concentration of promoter II was changed little after the first 4 days (Table 4). It had increased sharply by the eighth day after pollination. There was little difference in promoter II concentration between the 2 types of pollination throughout the collection period (Figure 11).

The promoter II concentration in the ovaries of unopened flowers in 1963 was only half that of the ovaries in unopened flowers in 1962 (Table 4). Four days after pollination in 1963 the concentration of promoter II in cross-pollinated ovaries had increased 100 percent while increasing only 50 percent in self- and non-pollinated ovaries. There was little change in concentration at 8 days in self- and cross-pollinated ovaries; however, the cross-pollinated ovaries maintained a slightly higher level. In non-pollinated ovaries on this date the promoter II concentration had dropped and was much lower than either the self- or cross-pollinated ovaries (Table 2 and Figure 11).

Promoter II concentrations in cross-pollinated ovaries rose through 12 days and by 16 days had increased twelvefold over the concentration on the date of pollination.

Because of the ovary abscission that occurred between 4 and 12 days after pollination there were no self- or non-pollinated ovaries available for sampling at 12 and 16 days.

Promoter III.-Promoter III, R_f 0.94 to 1.0 (section 17), was a minor area of promotion and it moved with the solvent front. The area of

the solvent front fluoresced a light purple under ultraviolet light though the color was not always confined to section 17. It sometimes extended through sections 16 and 15. There was no definite chromogenic reaction in this area with Ehrlich's reagent.

The low levels of promotion and inconsistent occurrence of promoter III throughout the experiment cast doubt on its existence as an actual growth-promoting substance. Similar peaks were observed when synthetic IAA was chromatographed alone. No further importance is assigned to this area of promotion.

Inhibitor.-A moderate to strong inhibitor occurred on all chromatograms assayed and was found to range from R_f 0.78 to 0.94 (sections 14 to 16) or R_f 0.83 to 0.94 (sections 15 and 16). An inconsistent array of colors in this area was observed over different chromatograms under ultraviolet light. These ranged from pink through yellow, ash, light blue, and light purple. These fluorescing areas were not always delineated by the areas of growth response. A pink, pinkish-brown, or yellow color was observed on some chromatograms treated with Ehrlich's reagent, while on others no color developed.

The concentration of the inhibitor on each chromatogram was determined as the difference between the units of growth of the control and the units of growth of those sections showing inhibition between R_f 0.78 and 0.94. The mean concentration for replicated bioassays of styles and ovaries is presented in Table 4 as units of inhibition per 0.25 gm fresh weight and on a per-style and per-ovary basis. The concentration of inhibitor per ovary is shown graphically in Figure 12.

There was an increase in concentration of the inhibitor throughout the sampling period, but it did not vary appreciably with year of sampling, pollen tube growth, or pollen source.

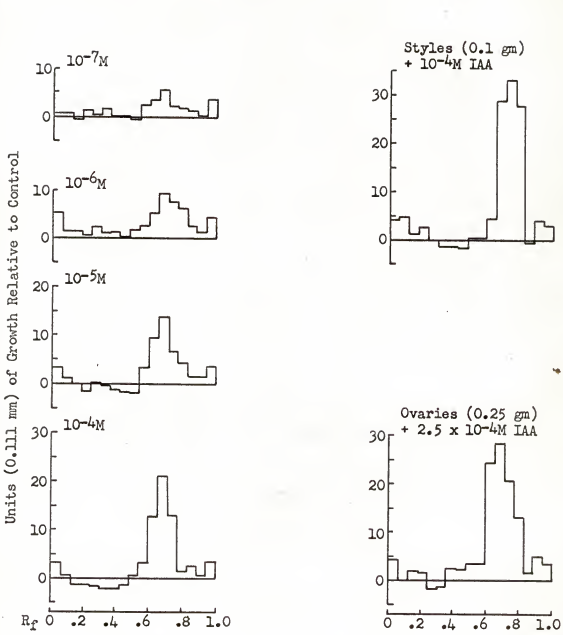

Figure 7 A. Histograms of 4 concentrations of IAA. Each histogram is the average of 2 independently developed chromatograms.

B. Histograms of chromatographed methanol extracts of styles and ovaries of unopened flowers plus IAA. Each histogram represents 1 chromatogram.

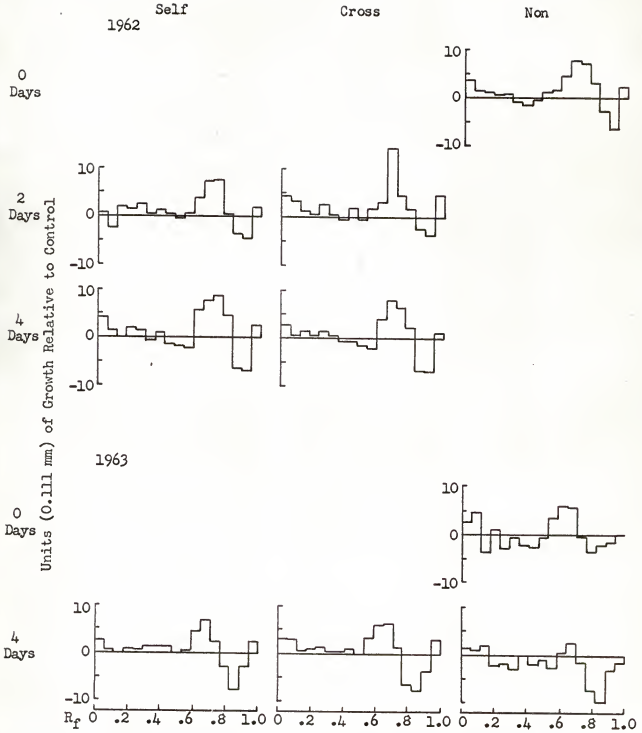

Figure 8. Histograms of chromatographed methanol extracts of styles collected at various dates following non-, self-, and cross-pollination in 1962 and 1963.

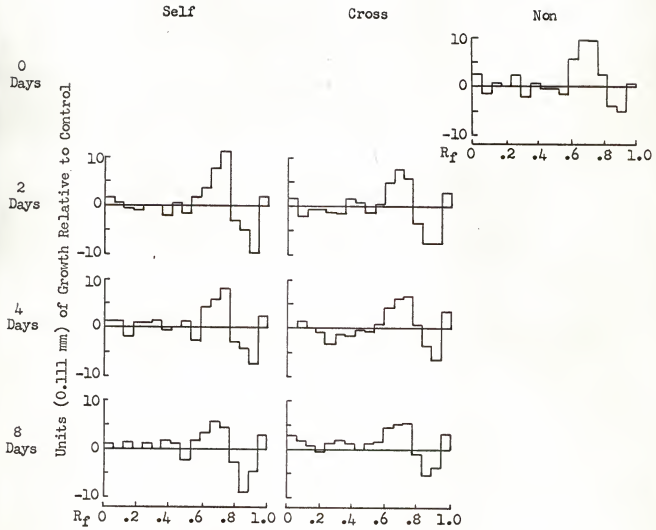

Figure 9. Histograms of chromatographed methanol extracts of ovaries collected at various dates following non-, self-, and cross-pollination in 1962.

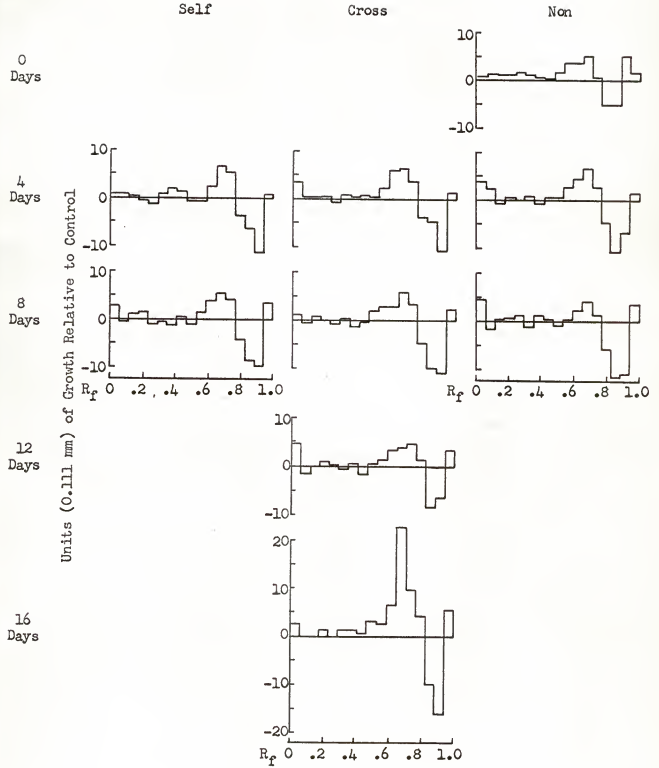

Figure 10. Histograms of chromatographed methanol extracts of ovaries collected at various dates following non-, self-, and cross-pollination in 1963.

TABLE 4.--Concentrations of promoter II (R_f 0.53-0.83) in ovaries and styles of 'Orlando' tangelos at various dates following non-, self-, and cross-pollination.[a]

Days After Pollination	Units per 0.25 gm Fresh Weight						Units per Individual Style or Ovary					
	Styles			Ovaries			Styles			Ovaries		
	Non	Self	Cross	Non	Self	Cross	Non	Self	Cross	Non	Self	Cross
1962												
0	22.2	-	-	26.2	-	-	2.01	-	-	2.10	-	-
2	-	20.9	24.3	-	24.4	19.1	-	1.90	2.32	-	2.22	1.82
4	-	26.7	20.2	-	18.1	17.7	-	3.05	2.31	-	2.26	2.22
8	-	-	-	-	15.3	17.1	-	-	-	-	3.22	3.60
1963												
0	18.6	-	-	13.4	-	-	1.73	-	-	1.05	-	-
4	4.9	14.3	14.6	16.7	15.1	17.8	0.52	1.55	1.54	1.52	1.63	2.04
8	-	-	-	8.6	13.9	14.7	-	-	-	0.90	1.55	1.84
12	-	-	-	-	-	13.1	-	-	-	-	-	2.50
16	-	-	-	-	-	45.1	-	-	-	-	-	12.00

[a]Concentrations expressed in units (unit/0.111 mm) of growth above controls.

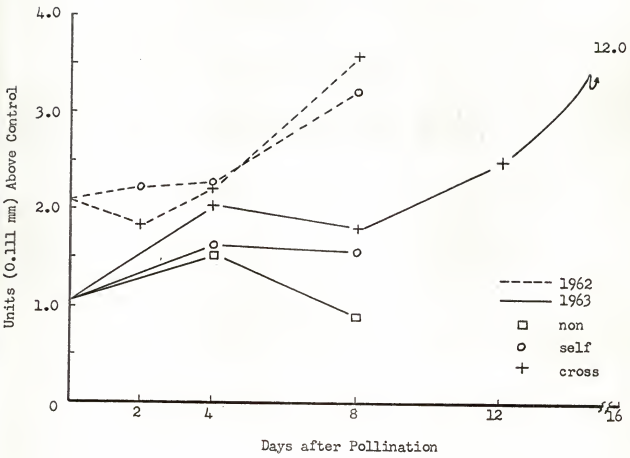

Figure 11. Concentration of promoter II (R_f 0.53-0.83) per ovary at various dates following non-, self-, and cross-pollination. Concentrations expressed in units (unit/0.111 mm) of growth above control.

TABLE 5.--Concentrations of inhibitor (R_f 0.78-0.94) in ovaries and styles of 'Orlando' tangelos at various dates following non-, self-, and cross-pollination.[a]

Days After Pollination	Units per 0.25 gm Fresh Weight						Units per Individual Style or Ovary					
	Styles			Ovaries			Styles			Ovaries		
	Non	Self	Cross	Non	Self	Cross	Non	Self	Cross	Non	Self	Cross
1962												
0	8.6	-	-	9.0	-	-	0.78	-	-	0.72	-	-
2	-	8.8	6.3	-	18.1	17.7	-	0.80	0.60	-	1.64	1.68
4	-	12.7	12.9	-	14.1	10.0	-	1.45	1.47	-	1.76	1.25
8	-	-	-	-	15.8	9.7	-	-	-	-	3.32	2.04
1963												
0	10.8	-	-	7.4	-	-	1.00	-	-	0.58	-	-
4	23.8	14.2	17.7	22.1	21.1	18.9	2.50	1.54	1.86	2.01	2.28	2.16
8	-	-	-	27.4	21.7	24.5	-	-	-	2.89	2.41	3.06
12	-	-	-	-	-	14.6	-	-	-	-	-	2.78
16	-	-	-	-	-	25.7	-	-	-	-	-	6.85

[a]Concentrations expressed in units (unit/0.111 mm) of growth below controls.

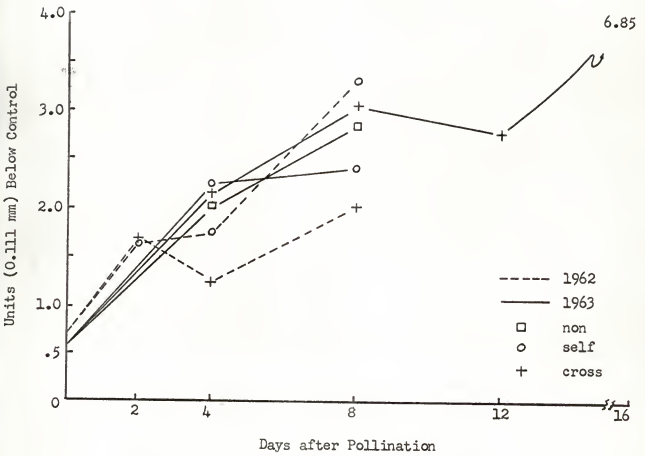

Figure 12. Concentration of inhibitor (R_f 0.78-0.94) per ovary at various dates following non-, self-, and cross-pollination. Concentrations expressed in units (unit/0.111 mm) of growth below control.

DISCUSSION

Morphological

Microscopic examinations of squashes of 'Orlando' tangelo pistils, following self- and cross-pollination, revealed that the pollen grains germinated and that large numbers of pollen tubes grew at equal rates to the base of the style and the central axis of the upper ovary. In this area the elongation of the 'Orlando' pollen tubes stopped. That 'Orlando' pollen tubes could reach this distance in approximately the same time as pollen tubes from cross-pollination shows that the factor or factors inhibiting the growth of 'Orlando' pollen tubes react at the base of the style or the upper ovary. These pollen tubes were never observed in the ovules.

These results are in contrast to the reported site of inhibition in the self-incompatible 'Clementine' tangerine. Oppenheimer (93) reported that the pollen tubes of 'Clementine' were apparently unable to grow beyond the tissue of the stigma and into the style after self-pollination. However, he implied that his technique may not have been adequate for observing pollen tube growth in the style. Self-incompatibility in many fruit crops, such as apple, pear, sweet cherry, and plum, has been reported to be due to slow growth of the pollen tubes in the style (16, 17, 24, 58, 65, 130), which is quite different than the type of incompatibility found in 'Orlando'.

The incompatibility reaction in 'Orlando' tangelo is similar to that of *Nemesia strumosa* and *Tolmiea menziesii* as described by Sears (112).

In those plants the pollen tubes were not inhibited until they reached the bottom of the style and they stopped growing at the top of the ovary. In Tolmiea menziesii only a small percentage of those tubes reaching the bottom of the style were able to grow into the ovary. Of the few tubes that entered the ovary, fewer yet reached the ovules.

Since the site of inhibition of the 'Orlando' pollen tubes is so near the ovules it is reasonable to assume that infrequently some pollen tubes may cross the area of inhibition and succeed in growing to the ovules. This could explain why, in solid-block plantings of 'Orlando' tangelos, fruit are occasionally found that contain 1 or 2 seeds. It may be that gene mutation in the male gametes causes the pollen tube to overcome the inhibiting barrier. Some workers have found that X-ray-induced mutations in the pollen of apple, cherry, and pear have resulted in the production of seeds after normally incompatible pollinations (66, 67).

The rate of pollen tube growth varied greatly between the 2 years covered by this study (Table 1). From what is known concerning the relation of temperature to pollen germination and tube growth, it is possible that the higher maximum and minimum temperatures that occurred the second year (Table 3) caused the more rapid tube growth that was observed (36, 56).

Following cross-pollination, pollen tubes penetrated the ovules and embryo sacs in a shorter time than had been reported by some workers for other citrus varieties (94, 32). However, the report by Ikeda (cited by Coit, 15) that fertilization occurred in from 2 to 3 days after pollination compares well with the 1963 observations in which 'Duncan' pollen tubes were seen in the embryo sacs 4 days after pollination.

Endosperm development was like that described for P. trifoliata

by Osawa (94). The endosperm nuclei were scattered throughout the embryo sac (Figure 3) and appeared to be attached to the thin-layered cytoplasm. Cytokinesis had not commenced up to 5 weeks after fertilization.

The 5-week period following fertilization, during which the zygote did not divide, is in agreement with the observations reported for other citrus varieties (32) and for P. trifoliata (94). Nucellar embryo development has been reported (32, 94) to begin near the time the zygote divides. This would explain why no nucellar embryo development was observed in this study.

Physiological

It has been a classical concept that there is a relationship between natural growth-regulating substances in plants and sexual reproduction (36, 43, 76). This concept recognized a hormonal stimulus associated closely with the processes of pollination, pollen tube growth, fertilization, and endosperm development (76). These hormonal stimuli have been considered responsible for initiation of fruit growth and subsequent development of fruit through maturity. Luckwill (76) has suggested that in the majority of fruits there is an initial stimulus required to initiate fruit development, with a further supply of hormonal substances required by the fruit through at least part of its development. Accordingly, the initial stimulus is provided by the developing pollen tubes and the subsequent stimuli come from other centers of auxin production, chiefly the developing seeds.

It has been shown that pollen and developing pollen tubes were associated with auxin production (64, 78, 83, 84, 126). It has also been found that developing ovules are associated with auxin production (5, 71, 86, 131).

In the work reported herein an attempt was made to detect any sequential changes in concentration of natural growth-substances in the styles and ovaries of 'Orlando' tangelo after pollination. An effort was made to collect samples as the pollen tubes were growing in the style, growing in the ovary, at the time of fertilization, and during embryo or endosperm development. In 1962 only self-pollinated and cross-pollinated flowers were collected. Because it was not known exactly when fertilization would take place, the sampling stopped short of this event. In 1963 non-pollinated flowers were also used and the sampling period was extended in an effort to include fertilization and subsequent ovule development. Because of more rapid pollen tube growth the second year, fertilization had already taken place by the first date of collection after pollination. Since the time relation was not the same for pollen tube growth and fertilization both seasons, certain inadequacies exist in the data. It is necessary, therefore, to discuss certain aspects of one season or the other and sometimes to draw upon the results of both seasons.

Because the fresh weight of the styles and ovaries increased during the sampling period and varied among self-, cross-, and non-pollinated flowers, it is considered more meaningful to discuss growth-promoter concentrations on a per-style or per-ovary basis. This procedure was followed in similar studies with tobacco (78, 79).

Relation of pollen tubes in the style to promoter II

The concentration of promoter II in the styles of unopened flowers on the day of pollination was approximately the same in 1962 and 1963 (Table 4). The 1962 collections taken 2 days after pollination were the only samples of styles that contained growing pollen tubes (Table 1).

In later samples the pollen tubes had already reached the base of the style or upper ovary.

In the 2-day samples in 1962, the concentrations of promoter II in both selfed and crossed styles were approximately the same and were relatively unchanged from that of the unopened flowers. Since the 2-day samples did not include non-pollinated styles it could not be concluded from these results that promoter II production was stimulated by growing pollen tubes. However, in 1963, non-pollinated styles were sampled and the concentration of promoter II was found to be only one-third as much as in either the styles of unopened flowers or the pollinated styles. From this evidence it is concluded that pollen tubes in the style did stimulate the production of promoter II. This conclusion is supported by similar results found with other plants (78, 83, 84).

Flowers collected on the day of pollination were unopened and therefore growth of the styles probably was not yet complete. Table 2 shows that the styles did increase in fresh weight after pollination. Therefore, the concentration of promoter II in the styles of unopened flowers in both 1962 and 1963 may have been related to the final growth taking place. Assuming this to be true, the auxin concentration would be expected to drop, perhaps to the level observed in the 4-day-old unpollinated styles, after growth of the styles was completed.

It is unlikely that the absence of pollen grains on the non-pollinated styles was the reason for the lower concentration of promoter II at 4 days in 1963, although pollen grains are known to contain growth hormones (56). Van Overbeek (126) has shown that the amount of pollen on a stigma is probably too low to contain any appreciable amount of endogenous auxin.

The styles did not begin to abscise until 8 days after pollination in 1962; however, at this same collection date in 1963, the styles had already abscised (Table 1). This difference in the time of style abscission may have been related to the fact that a higher concentration of promoter II was detected in the 4-day samples in 1962 than in 1963, thus prolonging style abscission in 1962.

Relation of pollen tubes in the style to promoter II in the ovary

In 1962 it could not be determined whether pollen tubes in the styles caused an increase in promoter II in the ovaries, since there were no non-pollinated pistils with which to make comparisons. However, the increase in ovary size prior to pollen tube entry into the ovary (Table 2) suggests that ovary growth was being stimulated by growing pollen tubes. In 1963 non-pollinated pistils were included, but the pollen tubes grew much faster than in 1962 and had already penetrated the ovary by the first sampling date after pollination. Thus the results do not give conclusive evidence that pollen tubes growing in the styles cause a build-up of promoter II in the ovary.

Relation of pollen tubes in the ovary to promoter II

On the eighth day after pollination in 1962, the pollen tubes of both 'Orlando' and 'Duncan' pollen had entered the ovaries but fertilization had not yet occurred (Table 1). At this date the concentration of promoter II in both selfed and crossed ovaries had risen sharply from the previous sampling date when the pollen tubes were in the style but not in the ovaries (Table 4). Despite the fact that there were no non-pollinated pistils for comparison, this evidence indicates that the presence of pollen tubes in the ovaries caused an increase in promoter II.

Similar results were reported by Lund (78), who found a build-up of IAA in tobacco ovaries after they were penetrated by pollen tubes.

In 1963 there was some evidence to the contrary, in that no difference was found between the concentration of promoter II in self- and non-pollinated ovaries on the fourth day after pollination although pollen tubes were in the self-pollinated ovaries. However, 8 days after pollination, the self-pollinated ovaries had a much higher concentration of promoter II than non-pollinated ovaries (Table 4). In the cross-pollinated ovaries fertilization had already taken place, therefore, they could not be used in these comparisons. While there are some inadequacies in the data, there is evidence that the presence of pollen tubes in the ovaries increased the concentration of promoter II.

Relation of fertilization to promoter II

The 1962 sampling did not extend through the time of fertilization. In 1963 pollen tubes had entered the embryo sacs in the cross-pollinated ovaries by 4 days after pollination (Table 1) and the concentration of promoter II was higher than in either self- or non-pollinated ovaries on this date (Table 4). The differences in concentration were small and it can only be assumed that this was due to fertilization. Workers have shown that in a number of plants there is a build-up in endogenous auxin concentrations following fertilization (5, 47, 89, 90, 131).

Relation of endosperm development to promoter II

In 1963 the pollen tubes had penetrated the embryo sac 4 days after pollination and endosperm development was observed 8, 12, and 16 days after pollination. There was a considerable increase in the concentration of promoter II in the twelfth- and sixteenth-day samples of cross-pollinated

ovaries. This is evidence that the developing endosperm increased the level of promoter II, an assumption that is substantiated by reports of other workers (5, 47, 51, 71, 72, 86, 119, 121).

Relation of promoter II to ovary abscission

Low promoter II concentrations appeared to be related to the ovary abscission that occurred between the fourth and twelfth day after pollination in 1963 (Table 4, Figure 6). Ovary abscission did not occur through 19 days after pollination in 1962 and it is assumed that this was due to the higher promoter II concentrations in these ovaries, although the last sampling date for physiological study in 1962 was 8 days after pollination.

Ovary abscission was less among cross-pollinated ovaries than either self- or non-pollinated ovaries in 1963 and this appears to have been due to the difference in promoter II concentrations. The large increase in concentration of promoter II in the cross-pollinated ovaries 16 days after pollination could have been related to the retention of ovaries between the sixteenth and fortieth day after pollination.

It may be hypothesized that promoter II is related to the abscission of young ovaries, and that as long as optimum concentrations are maintained, young ovaries remain attached to the tree. It is, therefore, suggested that the higher promoter II concentrations in 1962 caused the retention of ovaries on the trees, while the lower concentrations in 1963 resulted in the profusion of ovaries dropping from the trees.

Relation of pollen tube growth and promoter II to ovary enlargement and fruit set

The results suggest that pollen tubes in the style and ovary stimulated initial ovary enlargement. Ovaries 4 days after self- and

cross-pollination in 1963 had approximately 20 percent greater fresh weight than non-pollinated ovaries at this date (Table 2). It has been reported (126, 140, 141) that in some plants ovary enlargement was induced by pollination and was well under way before the pollen tubes reached the embryo sac. Pollen tube growth in the lower style and ovary, without fertilization, has actually enhanced fruit set in some plants (139, 140, 141). Luckwill (76) acknowledged that it was not unusual for some plants to develop parthenocarpic fruit following incompatible pollinations.

In pollination studies with 'Orlando', Krezdorn (60) found that non-pollinated flowers did not set fruit, but a small percentage of self-pollinated flowers set fruit which were seedless. This would indicate a slight stimulation resulting, not from fertilization, but from growth of the pollen tubes. It has been pointed out that the presence of pollen tubes in the ovaries increased the concentration of promoter II.

It is a reasonable hypothesis, therefore, that pollen tubes stimulate the production of promoter II and that this is a contributing factor in initial parthenocarpic fruit set in the 'Orlando'. It is further hypothesized that following cross-pollination, the multiplication of the endosperm nuclei causes a release of sufficient quantities of promoter II to maintain the auxin concentration above a critical level until sufficient auxin can be produced by the developing fruit. As long as an optimum auxin concentration is maintained, the fruits remain attached to the trees. If a low auxin level is present in the ovaries at the time of bloom, the additional auxin released during the growth of the pollen tubes may not be enough to maintain a sufficient auxin concentration unless fertilization and endosperm development occur.

A high initial concentration of promoter II in the pistils, enhanced

by the auxin produced by growing pollen tubes after self-pollination, may cause a good parthenocarpic fruit set. It is therefore hypothesized that the erratic parthenocarpic fruit set in 'Orlando' tangelo is the result of variations in the initial concentrations of endogenous promoter II.

This theory is supported by Luckwill (76), who suggested that there is a chemical stimulus accompanying pollination that initiates ovary growth, and a stimulus (or stimuli) originating in the seeds that is necessary for continued growth of the fruit to maturity. It has been pointed out by Gustafson (43) that some citrus varieties have reached the ultimate in parthenocarpy, i.e., fruit development has become completely independent of seed development. In such fruits auxin is presumably synthesized by the fruit tissue itself. Luckwill (74) described this type of fruit as an auxin autotroph. In the 'Orlando' tangelo it is suspected that the developing ovules enhance the supply of auxin produced by the developing fruit, inasmuch as many-seeded fruit attain larger sizes than do non-seeded or few-seeded fruit (60).

The critical period for initial fruit set in 1963 appeared to be between the fourth and twelfth day after pollination. It is reasonable to assume that this period would vary due to environmental factors such as temperature.

It has been shown that application of gibberellin to citrus flowers can increase fruit set (14, 61, 101, 118), although Krezdorn (61) noted that much of the fruit produced as a result of gibberellic acid applications was smaller than the naturally produced parthenocarpic fruit. It has been pointed out by Wittwer et al. (132) that in tomato IAA is relatively inactive in stimulating tomato fruit growth but shows a

marked synergism with gibberellin A_3. That gibberellins may enter into a synergistic relationship with promoter II or function in another fashion in citrus fruit set is a possibility; however, the presence of gibberellin could not be detected by the bioassay used in this study. There was no evidence that an endogenous stimulus other than promoter II was responsible for initial fruit growth.

Relation of external factors to promoter II in the pistils

The difference between the initial concentrations of promoter II in the ovaries of the unopened flowers in 1962 and 1963 may have been due to different conditions of the trees from which the sample material was taken. The 1963 samples were taken from trees which had endured sub-freezing temperatures 3 months previously and had lost approximately 5 percent of their leaves. Since leaves are recognized sites of auxin production it is possible that a reduction in leaf area reduced the natural auxin content of the flowers. The additional auxin required for replacement leaves plus the spring flush of growth could have contributed to the lower promoter II concentration detected in the ovaries of unopened flowers in 1963.

The trees were on rough lemon rootstock in 1962 and Cleopatra mandarin in 1963. The level of fertility of the soil and the nutritional status of the trees themselves were undetermined. It is unknown what effect these factors could have had on initial concentrations of promoter II.

Identifying characteristics of promoter II

Promoter II separated on the chromatograms at an R_f coincidental with that of IAA, whether the synthetic auxin was chromatographed alone or added to samples and carried through extraction concurrently. When

sufficiently high levels of promoter II occurred on chromatograms, a pink chromogenic reaction to Ehrlich's reagent was obtained. This color reaction was similar to that seen on IAA chromatograms treated with Ehrlich's reagent. Stowe et al. (121) reported that orange blossoms contained quantities of IAA. A recent report (57) has described an extractable hormone in the ovaries of Valencia and navel oranges that is not IAA although it separates at a similar R_f on isopropanol-ammonia-water chromatograms. The investigators have called the growth-substance "citrus auxin".

Relation of self-incompatibility to growth substances extracted

There was no indication that any of the growth substances extracted were responsible for self-incompatibility in the 'Orlando' tangelo. This does not mean that other extractable substances may not be related.

Other growth-promoting and growth-inhibiting areas detected on chromatograms

Because of the slight variation in the amount of the 2 minor areas of growth promotion, promoter I and promoter III, between types of pollination and over collection dates, a relationship to the progressive development of the pollen tubes or the setting of fruit could not be established.

The area of inhibition on the chromatograms ranging from R_f 0.78 to 0.94 was present in all samples and corresponded to that reported in sweet orange blossoms by others (121). These workers compared this inhibitor to that found in a number of plant tissues and termed inhibitor-B by Bennet-Clark and Kefford (9). Taylor (cited by Bentley et al., 10) was unable to associate the inhibition in this area with any specific compound. The variety of colors fluoresced under ultraviolet light

and developed with Ehrlich's reagent indicated that the inhibition found in the present study was not due to just one substance.

There was little variation in the amounts of inhibition between the different types of pollination. There was a slightly greater amount of inhibition in the non-pollinated ovaries and particularly the non-pollinated styles, indicating that this may be a degradation product. That it was responsible for fruit setting or failing to set was not indicated by the results, since the setting fruits also showed appreciable quantities of the inhibitor.

SUMMARY

1. Research was undertaken to determine the nature of sexual self-incompatibility in the 'Orlando' tangelo through a morphological study of pollen germination and pollen tube growth. A physiological study was made of the concurrent changes in natural growth substances in the pistils to determine their relation to the reproductive process and self-incompatibility. The morphological study was made with squashed and sectioned material. The physiological study was by paper chromatography and wheat straight growth biological assay.

2. A squash technique that allowed the observation of pollen tubes in pistils of citrus flowers is described.

3. 'Orlando' and 'Duncan' pollen germinated and pollen tubes grew at the same rate to the base of the styles and the upper ovary of the 'Orlando' pistils. Growth of the 'Orlando' pollen tubes was inhibited in this location and observations indicated that they never entered the ovules. 'Duncan' pollen tubes penetrated the 'Orlando' embryo sacs within 12 days after pollination in 1962 and within 4 days in 1963. This difference was attributed to higher field temperatures following pollination in 1963.

4. Endosperm development began within 2 days after pollen tubes were observed entering the embryo sac. The first division of the zygote had not taken place 5 weeks after fertilization.

5. On the chromatograms there was one major area of growth promotion, designated as promoter II, that separated at R_f 0.68. There was

one major area of growth inhibition that separated between R_f 0.78 and 0.94. Two minor areas of promotion, promoter I and promoter III, were located at the original streak and at the solvent front respectively. No significance is attached to the area of inhibition or the two minor areas of promotion.

6. Pollen tubes in the styles stimulated the production of promoter II. Evidence is presented which indicates that promoter II concentrations in the style may be related to abscission of the styles.

7. Evidence is presented which indicates that initial ovary enlargement is stimulated by pollen tubes growing in the pistil.

8. The hypothesis is presented that parthenocarpic fruit set in 'Orlando' tangelo is controlled by endogenous concentrations of promoter II and that greater fruit set following cross-pollination is due to the increased production of promoter II associated with endosperm development.

9. None of the growth-promoting or growth-inhibiting substances extracted were considered responsible for self-incompatibility in the 'Orlando' tangelo.

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Richard Albert Hensz was born July 17, 1929, in Evansville, Indiana. He received his elementary and secondary education in Indiana.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 18, 1964

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